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B cell epitopes of gliadin

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SUMMARY

A phage displayed dodecapeptide library and synthetic octapeptides spanning the complete sequence of α- and γ-type gliadin and overlapping in six amino acids (pepscan) were screened for binding to human gliadin antibodies (AGA). Phage display experiments led to four sequences recognized with significantly higher frequency by sera with raised IgA-AGA titres than by control sera. All these peptides contained the core sequence PEQ. Pepscan experiments revealed binding of AGA to five prominent regions: (i) QXQPFP (binding to IgG and IgA, X representing P, Q, and L); (ii) IPEQ (IgG) and WQIPEQ (IgA); (iii) FFQP (IgG) and QGXFQP (IgA, X representing F and S); (iv) PQQLPQ (IgG and IgA), all in α-type gliadin; and (v) QPQQPF (IgG and IgA) in γ-type gliadin. In two of the sequences (QPQQPF and QQQPFP), substitution of Q by E resulting in QPEQPF and QEQPFP, respectively, increased significantly binding of AGA from sera of patients with biopsy-proven or suspected coeliac disease (CoD), all positive for endomysium antibodies (EmA). In contrast, binding of sera with high AGA titre from EmA-negative patients (CoD and dermatitis herpetiformis excluded) was not enhanced by this substitution. Thus, AGA directed against these modified epitopes can be regarded as specific for CoD. This is the first study demonstrating that deamidation of gliadin improves reactivity of AGA of CoD patients.

Keywords coeliac disease epitopes gliadin antibodies pepscan phage display

INTRODUCTION

Coeliac disease (CoD) is an intestinal disorder induced in sensitive patients by gliadin from wheat and by prolamins from related cereals (for review see [1–4]). The characteristic lesion consists of villous atrophy and crypt cell hyperplasia and the mucosa, especially the epithelium, is densely infiltrated with lymphoid cells. There is a strong association with the HLA DQ2 and DQ8 haplotype and the number of DQ-restricted mucosal T cells is increased. Mucosal B cells are triggered to produce immunoglobulins against the offending food antigens (anti-gliadin antibodies (AGA)) and against self molecules (autoantibodies). Recently, tissue transglutaminase (tTG) was discovered as a major autoantigen [5]. However, the role of this enzyme in the pathogenesis is not yet completely understood. tTG may be responsible for linking gliadin to self-molecules [5,6] but the enzyme may also be involved in deamidation of glutamine residues in gliadin, as demonstrated *in vitro* [7,8]. Deamidation is important for binding of gliadin-derived peptides to HLA DQ2 and DQ8 molecules and subsequently for the stimulation of T cells [7–12]. Several gliadin-derived peptides were identified as ligands for the disease-associated HLA DQ molecules [7–9,11–13].

Contrary to T cell epitopes of gliadin, B cell epitopes are less well investigated. This may be due to the fact that AGA are produced not only in CoD but also in other conditions with damaged gut mucosa and thus are considered as unspecific. Until now there are only few reports [14–16] with divergent results concerning B cell epitopes of A-gliadin [17] and α/β-gliadin [18], members of the α-type gliadins, and there is no information at all about epitopes in γ-type gliadins. Therefore, we screened phage-displayed libraries as well as synthetic peptides with human AGA. We identified four prominent epitopes in α-type gliadin and one prominent and repetitive epitope in γ-type gliadins. In several positions of the epitopes, deamidation is important for binding of the antibodies.

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MATERIALS AND METHODS

Human sera

In 99 sera IgA-AGA were investigated. From these, in 58 sera AGA titre was $>$ 10 U/ml (106 $\pm$ 126 U/ml; high titre) and in 41 sera $<$ 10 U/ml (4·3 $\pm$ 2·8 U/ml; controls). In 31 sera, IgG-AGA were investigated including 14 sera with AGA titre $>$ 10 U/ml (129 $\pm$ 36 U/ml; high titre) and 17 sera with AGA titre $<$ 10 U/ml

(2·2 ± 2·8; controls). In experiments in which the group of patients with high IgA-AGA titres was divided into subgroups with and without endomysium antibodies (EmA), all EmA-positive patients had biopsy-proven CoD, suspected CoD, or dermatitis herpetiformis, and in all EmA-negative patients CoD or dermatitis herpetiformis was excluded. Absorption of AGA was performed by addition of gliadin [19] to sera (1 mg/ml) followed by 1 h of incubation at 4°C and centrifugation. The supernatant was absorbed again. After five cycles of absorption sera were tested for titre of AGA and EmA.

Isolation of IgA

For phage display experiments, IgA from human sera was isolated by affinity chromatography on an anti-human IgA column. Purity of isolated antibodies was analysed by SDS–PAGE [20] and Western blot [21].

Assay of AGA and EmA

IgA and IgG AGA were determined by enzyme immunoassay in microwells coated with gliadin as described previously [22]. IgA class EmA were determined by investigating their binding to monkey oesophagus sections (Virimmun, Frankfurt, Germany) [23].

Phage display and sequencing

The Ph.D.-12 Peptide 12-mer Library Kit (New England Biolabs, Schwalbach, Germany; Code 8110) was applied as described [22,24]. For screening, IgA fractions from four different EmA-positive sera with high AGA titre (mean 148 U/*l*) were used separately. After three rounds of selection with each IgA fraction, 72 clones were isolated and amplified. The DNA was isolated and cycle-sequenced using primers according to the instructions of the manufacturers. The 288 clones investigated contained inserts with 200 different sequences.

Pepscan

Octapeptides overlapping by six amino acids spanned the sequence (excluding the propeptide regions) of α/β-gliadin precursor (accession number C22364) [18] and of γ-gliadin precursor (accession number P21292) [25]. The peptides were prepared by automated spot synthesis [26] as previously described in detail [27] and were covalently bound to a cellulose membrane (Abimed, Langenfeld, Germany) via their C-termini. After washing in methanol and buffer (TBS–T: NaCl 137 mM, KCl 2·7 mM, Tris 50·4 mM, Tween 0·05%, pH 8·0), membranes were blocked, washed in TBS–T, and incubated in human serum (1:100). After further washing steps membranes were incubated with anti-human IgA or IgG conjugated with peroxidase (Dako, Hamburg, Germany; Code P216 or P214, respectively, 1:500 in TBS–T). After washing, luminescence was measured using Supersignal CL-HPR kit according to the instructions of the manufacturer (Pierce, Rockford, IL, Code 34080) on Hyperfilm-ECL (Amersham, Braunschweig, Germany). Binding was assessed qualitatively as strongly positive (score 2), positive (score 1), or negative (score 0). Recognition of the different peptides by patients' antisera was expressed as reactivity index (RI) calculated by subtraction of the mean score of control sera from the mean score of sera with high AGA titre.

Statistical analysis

Differences between recognition of peptides by sera with high AGA titres and control sera were calculated using Fisher's exact test.

RESULTS

Pepscan experiments showed that the profile of recognition of gliadins by antibodies was not uniform but showed distinct peaks (Fig. 1). In α-type gliadin, there were several reactive peptides in the N-terminal region, one prominent peak in the middle of the molecule, and three further peaks close to the C-terminus. In γ-type gliadin, reactivity was mainly found in the N-terminal part of the polypeptide chain. The reactivity of IgA and IgG antibodies was very similar.

For alignment of α-type gliadin peptides, all sequences recognized with a RI $> 0{\cdot}4$ were considered. Four consensus sequences were obtained (Table 1): QXQPFP for IgG and IgA (part of a repetitive sequence between positions 33 and 65), IPEQ for IgG and WQIPEQ for IgA (position 167–172), FFQP for IgG (position 241–244) and QGXFQP for IgA (positions 239–244 and 252–257), and PQQLPQ for IgG and IgA (position 256–262).

From the peptides spanning the γ-type gliadin sequence those were considered as major epitopes which were recognized with a RI $> 0{\cdot}6$. All of them could be aligned to one common consensus sequence QPQQPF for IgG as well as IgA epitopes (Table 1). The sequence QPQQPF is part of a repetitive sequence occurring in the N-terminal region (position 8–137) of γ-type gliadin. This explains multiple peaks of reactivity of human AGA in this part of γ-type gliadin (Fig. 1).

Screening a phage displayed dodecapeptide library with human IgA of EmA-positive sera with high AGA titre yielded 200 different sequences (peptide group A). Dodecapeptides with the respective sequences were synthesized on cellulose and IgA binding from 14 sera with high AGA titre and six control sera was examined in the luminescence assay. From the 200 peptides, 123 were not recognized at all or were recognized more often by control sera. The remaining 77 peptides (peptide group B) were retested for IgA binding with 24 sera with high AGA titre and with 21 sera with low AGA titre. Four peptides, VHFTMPEQPFRT, QPQPAVAFPEQD, YPPEQPGSAFPE, and HEPAPEQAWDPF, were recognized with significantly higher frequency by sera with high AGA titre ($P \leq 0{\cdot}055$) (peptide group C). All contained the sequence PEQ.

To investigate if PEQ is important for gliadin epitopes, those dodecapeptides of group B which contained the PEQ sequence were scanned with 17 sera. For that, hexapeptides were applied overlapping by five amino acids. The reactivity of IgA was nearly solely due to the PEQ sequence (Fig. 2).

However, PEQ was found only in one of the five consensus sequences obtained by pepscan experiments with α- and γ-type gliadins. Therefore, the third position in the heptapeptide QPQQPF, constituting the consensus sequence recognized in γ-type gliadin, was substituted by different amino acids and binding of IgA of three different EmA-positive sera with high AGA titre was investigated in the luminescence assay. In all of them, substitution of Q by E or D resulted in a strong increase in reactivity, whereas substitution analogues with positively charged amino acids like R and K and with aromatic amino acids like W and Y were not recognized at all (Fig. 3).

PQQLPQ represents another sequence recognized by AGA in pepscan experiments (Table 1). This sequence can also be converted into a PEQ containing peptide by a Q→E substitution.

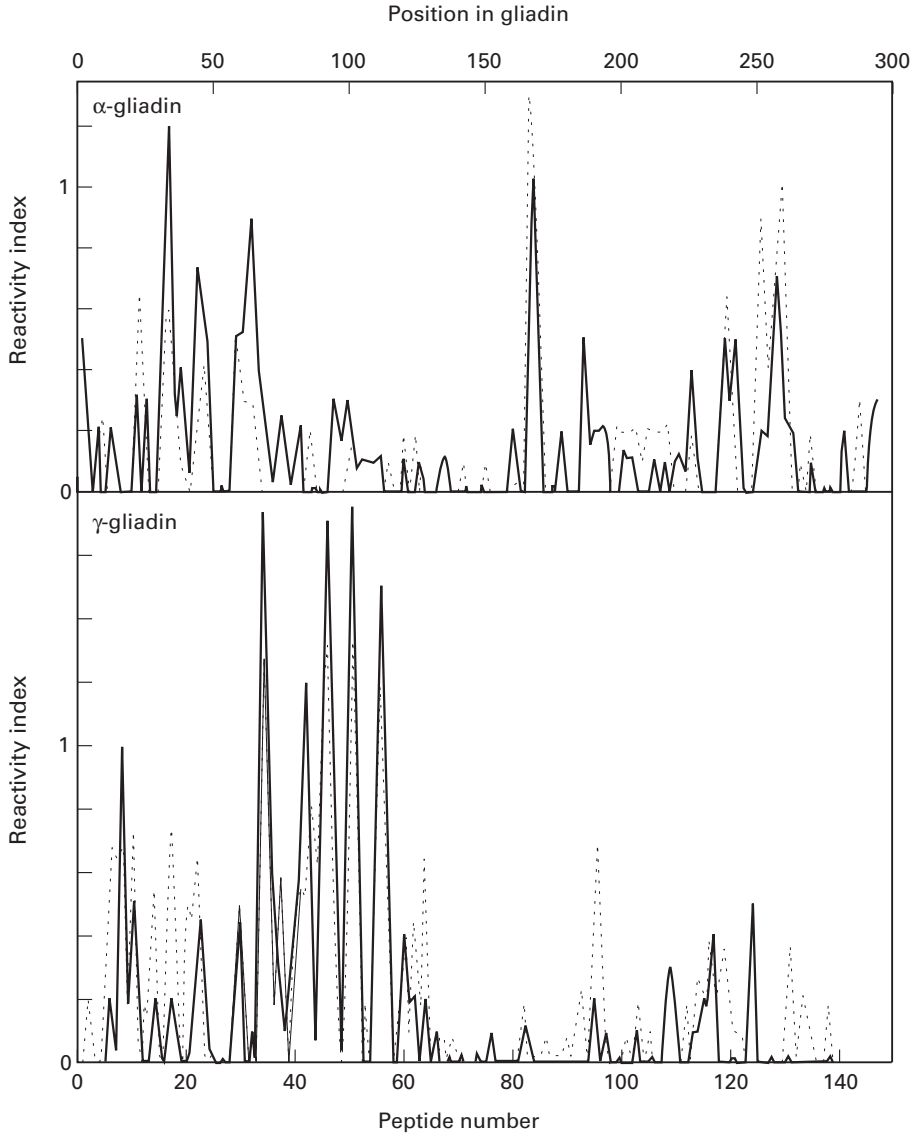

Fig. 1. Reaction profile of human immunoglobulins over gliadin sequence. ——, IgG; · · · · ·, IgA. α-type gliadin sequence was tested using sera with high AGA titre (IgA 252·5 ± 171·0 U/ml, IgG 131·5 ± 37·2 U/ml) *versus* control sera (AGA titres, IgA 3·9 ± 2·8 U/ml, IgG 1·8 ± 2·7 U/ml) and γ-type gliadin sequence using sera with high AGA titre (IgA 214·1 ± 176·5 U/ml, IgG 122·1 ± 37·8 U/ml) *versus* control sera (AGA titres, IgA 3·8 ± 2·7 U/ml, IgG 2·8 ± 2·9 U/ml). Number of sera in each group ≥ 10.

Therefore, the peptides PQQLPQ, PEQLPQ together with QPQQPF and QPEQPF were tested in the luminescence assay using sera with high AGA titre and control sera. Two subgroups of sera with high AGA titre were investigated, consisting of EmA-positive and EmA-negative individuals. Interestingly, enhanced activity after Q→E substitution was only observed for sera of EmA-positive patients with high AGA titre and only with the peptide QPEQPF but not with the sequence PEQLPQ (Table 2).

Reactivity with the deamidated form of the peptides cannot be due to EmA also being present in the sera investigated, as shown by results from experiments in which sera were absorbed with gliadin to remove AGA. After absorption of AGA, the reactivity with the peptides in the amidated as well as in the deamidated form was clearly decreased or even abolished, although the titre of EmA was not affected by the absorption process (Table 3).

Finally, Q residues in all peptides carrying the consensus sequences obtained from pepscan experiments (Table 1) were substituted in all possible combinations by E and recognition by 10 EmA-positive sera (same as in Table 1) was investigated in the luminescence assay. For only two of these peptides did Q→E substitution result in an enhanced binding of IgA (third position in QPQQPF and second position in QQQPFP, $P < 0{\cdot}05$). Substitutions in other positions and in other peptides did not influence antibody recognition or even decreased it.

DISCUSSION

Currently the knowledge about B cell epitopes in α-type gliadins is still limited. Until now, in three studies overlapping synthetic peptides spanning the sequence of A-gliadin [17] or α/β-gliadin [18] were tested for reactivity with human sera [14–16] and the results are divergent (Fig. 4). In this study we demonstrate that

Table 1. Sequences recognized by AGA in pepscan experiments

No.	Sequence	Gliadin	Sequence found in peptide number	Region in gliadin	Immunoglobulin class	RI_{max}
1	QXQPFP	α	16,17,19,22–24,29,31–33	31–72	IgG	1·20
	QXQPFP	α	16,17,29	31–64	IgA	0·60
2G	IPEQ	α	83–85	165–176	IgG	1·02
2A	WQIPEQ	α	83–85	165–176	IgA	1·20
3G	FFQP	α	119,121	237–248	IgG	0·50
3A	QGXFQP	α	119,120,125,126	237–258	IgA	0·90
4	PQQLPQ	α	128,129	255–264	IgG	0·70
	PQQLPQ	α	128–130	255–264	IgA	1·00
5	QPQQPF	γ	8,34,35,41,42,45–47,50–52,55–57	15–120	IgG	1·70
	QPQQPF	γ	6,10,17,20,34,35,40,42,43,45,46,50, 51,55,56	11–118	IgA	1·20

Sequences obtained by aligning all peptides with reactivity index (RI) $>$ 0·4 (α-type gliadin) or $>$ 0·6 (γ-type gliadin) except four peptides of α-gliadin (peptide 1: VRVPVPQL, recognized by IgG with RI = 0·52, peptide 11: QVPLVQEQ, recognized by IgA with RI = 0·6, peptide 12: PLVQEQQF, recognized by IgA with RI = 0·5, and peptide 93: ILHQQHH, recognized by IgG with RI = 0·5).

reactivity of human AGA is not equally spread over the complete gliadin sequence but is confined to distinct regions. Our data are in agreement with previous results on the location of antibody recognition sites in the N-terminal repetitive region [14,15]. The peptide QXQPFP (X for Q, P, or L) is found in the sequences reported by both groups in identical or homologous manner and occurs four times in the N-terminal repetitive region. There is one further sequence (QPQPFR) in the N-terminal repetitive region homologous to QXQPFP. In accordance with the other three reports, reactivity can also be found in the central and C-terminal part of α-type gliadin but the position of the identified peptides is different. The sequence QGXFQP (X for S or F) occurs twice in the C-terminal region of α-type gliadin. The differences from other reports may be due to the different size of the peptides used and to the different number of overlapping amino acids. Furthermore, in one report [16] the interaction of peptides with AGA was investigated in a competitive assay which might be compromised by different specificity of AGA.

Our results provide first information about the location of B cell epitopes in γ-type gliadin. In contrast to α-type gliadin, reactivity of AGA with γ-type gliadins is clearly restricted to the repetitive region close to the N-terminus. The identified consensus sequence QPQQPF is present five times in γ-type gliadin and there are at least four additional repeats comprising the homologous sequences QPQQTF, QPQQTY, or QPQLPF. Furthermore, the peptide QXQPFP typical of α-type repetitive sequences is contained once in the N-terminus of γ-type gliadin.

Occurrence of B cell epitopes of AGA in the repetitive N-terminal region of gliadins may be caused by two factors. First, the N-terminal repetitive regions both in α- and γ-type gliadin form regularly repeated β-turns [28,29], which are usually located at the surface of proteins and therefore may function as antigenic sites [29]. β-turns are also found in oligopeptides which arise after digestion of gliadin. Second, immunoselection may favour such B cells which produce most efficient antibodies, i.e. immunoglobulins against epitopes occurring repetitively.

In addition to pepscan experiments, antibody reactivity was investigated by the phage display approach. Whereas all of the four peptides obtained from phage display experiments contained the PEQ sequence, PEQ was found in only one (WQIPEQ) out of five sequences when using synthetic peptides (Table 1). From previous reports it is known that glutamine residues in gliadin can be deamidated by the action of tTG [7] or by acidic treatment [9,10]. Deamidation of gliadin peptides was previously shown to

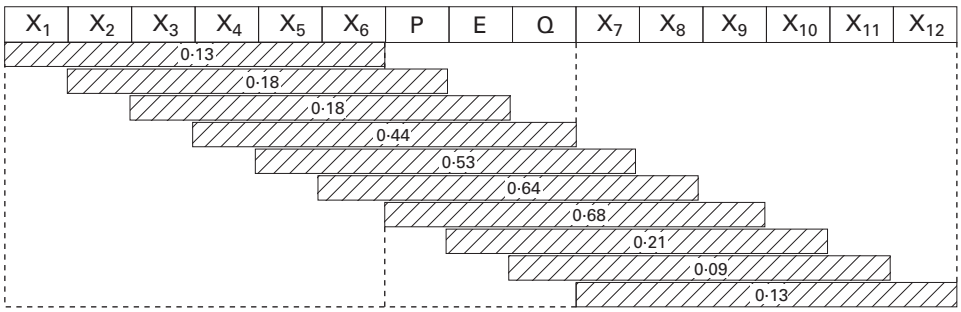

Fig. 2. Scan over dodecapeptides containing the PEQ sequence. Nine different representatives of dodecapeptide group B containing the PEQ sequence were scanned by overlapping hexapeptides spotted to cellulose membranes. The hexapeptides were screened with 15 sera with high AGA titre (77·2 ± 124·0 U/ml) for binding of IgA. The hexapeptides were aligned according to the position of PEQ. Results as mean scores. The reactivity of antibodies was nearly solely due to the PEQ sequence.

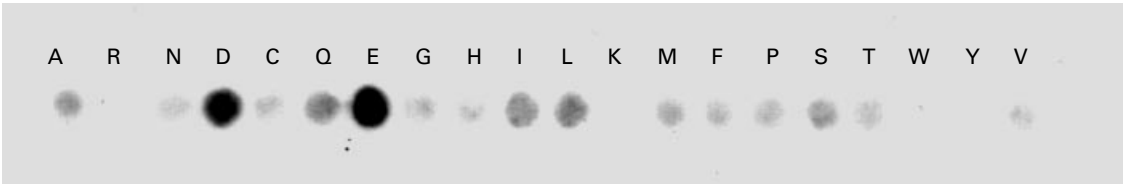

Fig. 3. Position 3 in peptide QPQQPF was substituted by different amino acids as indicated and reactivity of a single coeliac patient serum, representative of three examined, was investigated in luminescence assay. The peptides with E and D reacted more strongly than that with Q. Peptides with positively charged amino acids R and K and with aromatic amino acids W and Y were not recognized.

be important for binding to DQ2 molecules [7–12]. Moreover, our data show for the first time that deamidation of gliadin peptides is essential for binding to gliadin antibodies. Comparable to the generation of T cell epitopes [7–9], only Q→E substitutions at distinct positions result in enhanced binding of IgA, which may be due to the sequence specificity of tTG.

Due to deamidation, the sequence of native gliadin peptides used for pepscan studies does not reflect the sequence predominating at the site of antibody production in the gut mucosa. This may be a further important reason for divergent results of pepscan studies investigating epitopes of AGA.

Moreover, our results suggest that AGA may not represent an epiphenomenon of CoD but may play a decisive role in the delivery of gliadin peptides into antigen processing cells of the gut mucosa. After binding of gliadin to membrane-bound B cell receptors or to soluble IgG or IgA antibodies which in turn can associate with Fc receptors, gliadin could be taken up and delivered for intracellular processing. The subsequent presentation by DQ2 molecules would initiate a more efficient T cell response. Repetitive epitopes may represent an important prerequisite for cross-linking of Fc and B cell receptors and thus for activation of B and accessory cells.

Interestingly, three of the five B cell epitopes (Table 1) are found as identical or homologous sequences in peptides reported as ligands of HLA DQ2 molecules and in T cell epitopes: QXQPFP in α-gliadin 31–37 [13], QGXFQP (and FFQP) in the minimal peptide of α-gliadin 198–222 [11], and QPQQPF in the minimal peptide of γ-gliadin 134–153 [7,9,12]. This points to a role of B cell epitopes as targets for immunoglobulins for delivery to MHC molecules.

The finding that only IgA of EmA-positive but not of EmA-negative patients with high AGA titre showed an enhanced recognition of deamidated B cell epitopes cannot be due to cross-reactivity between AGA and coeliac autoantibodies. Lack of such cross-reactivity was demonstrated already earlier [30–32]. In accordance with previous results, our experiments indicate that binding to the deamidated epitopes is correlated with AGA but not with EmA (Table 3). Further, the short sequences PEQ and QEQ recognized by coeliac sera are not found in the sequence of human tTG [33]. Instead of cross-reactivity, our finding of enhanced binding after Q→E substitution may be due to an increased activity of tTG in CoD. Thus, the existence of CoD-specific B cell epitopes in gliadin can be proposed.

Pepscan and phage display are better suited to localize linear than conformational epitopes. Even if several of the phage peptides bear a sequence mimicking a conformational epitope, this cannot be aligned to gliadin sequence. Due to intraluminal and mucosal digestion however, persistence of large fragments of gliadin able to form conformational epitopes remains to be shown.

Table 2. Reactivity of AGA with two gliadin epitopes and their partially deamidated counterparts

	AGA^+ EmA-positive patients†	AGA^+ EmA-negative patients‡	Healthy blood donors§
QPQQPF	0·60 ± 0·52	0·55 ± 0·82	0·00 ± 0·00
QPEQPF	1·80 ± 0·42*	0·45 ± 0·82	0·20 ± 0·63
PQQLPQ	0·20 ± 0·42	0·55 ± 0·93	0·00 ± 0·00
PEQLPQ	0·20 ± 0·42	0·36 ± 0·67	0·20 ± 0·63

Peptides spotted to cellulose membranes were incubated with human sera and binding of IgA estimated in luminescence assay. Results as mean scores ± s.d. of ≥ 10 sera. Score range 0–2.

†Seven patients with biopsy-proven and three with suspected coeliac disease (CoD), mean AGA titre 180 U/ml.

‡CoD and dermatitis herpetiformis excluded, mean AGA titre 143 U/ml.

§Mean AGA titre 2·9 U/ml.

*$P < 0{\cdot}05$ compared with QPQQPF.

Table 3. Reactivity of three gliadin-absorbed sera with peptides representing two gliadin epitopes and their partially deamidated counterparts in luminescence assay (see Table 2)

		Serum no.		
	Absorption	513	971	1527
IgA-AGA titre	before	1382	262	196
	after	128	6·4	0·0
IgA-EmA titre	before	1:1280	1:320	1:160
	after	1:1280	1:320	1:160
Reactivity* with QPQQPF	before	2	1	0
	after	1	0	0
QPEQPF	before	3	3	2
	after	1	1	0
PQQLPQ	before	0	0	0
	after	0	0	0
PEQLPQ	before	0	0–1	0
	after	0	0	0

*Score range 0–3.

Fig. 4. Peptides recognized by human gliadin antibodies. Line (A), sequence of α-type gliadin [18], double line, peptides recognized by IgA in the present study; single line, sequence highly homologous with QXQPFP; line (B), screening for IgG binding [14]; line (C), screening for IgA and IgG binding [15]; line (D), screening with affinity-purified AGA [16]: [1]most active part of peptide A4; [2]overlapping peptides C2 and C3; [3]overlapping peptides F3 and F4.

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